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Molecular basis for the inhibition of human α-thrombin by the macrocyclic peptide cyclotheonamide A

(serine protease/α-keto amide/transition-state analogue/x-ray crystallography/total synthesis)

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ABSTRACT **The macrocyclic peptide cyclotheonamide A (CtA), isolated from the marine sponge *Theonella* sp., represents an unusual class of serine protease inhibitor. A complex of this inhibitor with human α-thrombin, a protease central to the bioregulation of thrombosis and hemostasis, was studied by x-ray crystallography. This work (2.3-Å resolution) confirms the structure of CtA and reveals intimate details about its molecular recognition within the enzyme active site. Interactions due to the "Pro-Arg motif" (Arg occupancy of the S_1 specificity pocket; formation of a hydrogen-bonded two-strand antiparallel β-sheet with Ser^{214}–Gly^{216}) and the α-keto amide group of CtA are primarily responsible for binding to thrombin, with the α-keto amide serving as a transition-state analogue. A special interaction with the "insertion loop" of thrombin (Tyr^{60A}–Thr^{60I}) is manifested through engagement of the hydroxyphenyl group of CtA with Trp^{60D} as part of an "aromatic stacking chain." Biochemical inhibition data (K_i values at 37°C) were obtained for CtA with thrombin and a diverse collection of serine proteases. Thus, CtA is just a moderate inhibitor of human α-thrombin (K_i = 0.18 μM) but a potent inhibitor of trypsin (K_i = 0.023 μM) and streptokinase (K_i = 0.035 μM). The relative lack of potency of CtA as a thrombin inhibitor is discussed with respect to certain structural features of the enzyme complex. We also report the total synthesis of CtA, by a convergent [2 + 3] fragment-condensation approach, to serve the preparation of cyclotheonamide analogues for structure–function studies.**

Thrombin (EC 3.4.21.5) is a trypsin-like serine protease present in blood and central to the bioregulation of thrombosis and hemostasis (1–3). Among its multitude of functions, thrombin converts fibrinogen into clottable fibrin, potently stimulates platelet aggregation, activates several coagulation factors, serves as a potent mitogen for diverse cell types, and even modulates brain function (1–6). Thus, there has been considerable interest in the search for natural and synthetic thrombin inhibitors, which hold significant potential as therapeutic agents, and in molecular mechanisms of thrombin inhibition (3).

Cyclotheonamide A (CtA; Fig. 1*a*), a macrocyclic pentapeptide isolated from the Japanese marine sponge *Theonella* sp., is reported (8) to inhibit thrombin with an IC_{50} value of 0.1 μM. The discovery of CtA has been particularly exciting (7–9) for several reasons. First, macrocyclic serine protease inhibitors, exclusive of large proteins such as pancreatic trypsin inhibitor (10), are rare in nature. Second, CtA contains a vinylogous tyrosine unit, which constitutes an example of a class of interesting peptide-like building blocks (7, 9). Third, it is possible that the α-keto amide group of CtA is poised to act as a "transition-state analogue," by interaction of the electrophilic ketone carbonyl (C^2; Fig. 1*a*) with the hydroxyl group of Ser^{195}, part of the thrombin catalytic triad His^{57}-Asp^{102}-Ser^{195} [chymotrypsinogen numbering (11, 12)], to generate a tetrahedral intermediate (13–15). Also, CtA contains a Pro-Arg structural motif that correlates with the P_2-P_1 positions in the tripeptide class of thrombin inhibitors, represented by D-Phe-Pro-Arg-CH_2Cl (PPACK) (16, 17) and Me-D-Phe-Pro-Arg-H (GYKI-14766) (18, 19) (Fig. 1*b*).

Given the crucial role that enzyme transition-state analogues have played in understanding enzyme mechanisms and in the practice of drug design (13, 20–22), we have pursued a multidisciplinary study of CtA and its analogues. Here we present results from x-ray crystallography, which define the molecular basis for inhibition of human α-thrombin by CtA, and the inhibition profile of CtA with 10 diverse serine proteases. Moreover, we report the total synthesis of CtA by a synthetic regime suitable for the preparation of diverse analogues.‖

MATERIALS AND METHODS

Materials. Human α-chymotrypsin and bovine trypsin were obtained from Sigma; other enzymes were obtained from American Diagnostica (Greenwich, CT). *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (pNA) was obtained from Sigma; other substrates were obtained from American Diagnostica. Hirugen (*N*-acetyl hirudin 53–64) was kindly supplied by John Maraganore (Biogen); human α-thrombin for crystallography was kindly supplied by John W. Fenton II (New York State Department of Health, Albany). Natural CtA (monotrifluoroacetate hydrate) from N. Fusetani (8) was used for crystallography. Argatroban (65:35 diastereomeric mixture; monohydrate) was kindly provided by Mitsubishi Kasei Corp. GYKI-14766 (monotrifluoroacetate dihydrate) was prepared by M. Costanzo (R. W. Johnson Pharmaceutical Research Institute), according to a published procedure (18).

Enzyme Assays and Measurement of Kinetic Constants. Enzyme-catalyzed hydrolysis rates were measured spectrophotometrically by using commercial enzymes and chromogenic substrates [Molecular Devices (Palo Alto, CA) microplate reader, 37°C]. Changes in absorbance at 405 nm were monitored with and without inhibitor. Data were ana-

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Abbreviations: CtA, cyclotheonamide A; PPACK, D-Phe-Pro-Arg-CH_2Cl; pNA, *p*-nitroanilide; CBZ, carbobenzoxy.

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‖The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (no. 1TMB).

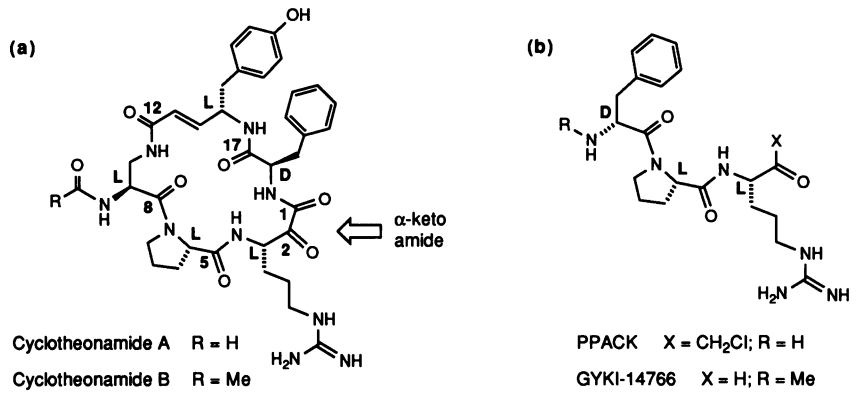

FIG. 1. Chemical formulas. (*a*) Cyclotheonamides A (R = H) and B (R = Me), with ring numbering and α-keto amide (C^1-C^2) denoted. The structural formula is revised stereochemically (7) from that originally reported with respect to C^{15}. (*b*) Two reference thrombin inhibitors, PPACK (D-Phe-Pro-Arg-CH_2Cl) and GYKI-14766 (Me-D-Phe-Pro-Arg-H).

lyzed with SOFTmax (Molecular Devices) and K·CAT (Bio Metallics, Princeton, NJ). Each reaction was started with addition of enzyme, and progress was followed for 30 min; initial rates of hydrolysis were determined from the linear portion of the saturation-kinetics plots. Substrates, conditions, and enzymes (total vol, 200 μl) were as follows: 10–100 μM Spectrozyme TH [*H*-D-hexahydrotyrosyl (HHT)-Ala-Arg-pNA·2AcOH]/10 mM Tris/10 mM Hepes/500 mM NaCl/0.1% polyethylene glycol (pH 7.85) for human α-thrombin (0.125 NIH unit/ml); 0.05–1.0 mM Spectrozyme tPA [CH_3SO_2-D-cyclohexyltyrosyl (CHT)-Gly-Arg-pNA·AcOH]/0.03 M Tris/0.03 M imidazole/0.2 M NaCl (pH 8.4) for human two-chain tissue-type plasminogen activator (723.8 international units/ml); 0.05–1.0 mM Spectrozyme P Kal (*H*-D-Pro-HHT-Arg-pNA)/0.05 M Tris/0.01% Tween 80 (pH 7.8) for human kallikrein (0.025 unit/ml); 0.05–1.0 mM Spectrozyme UK [carbobenzoxy (CBZ)-(γ)Glu(α-*t*-BuO)-Gly-Arg-pNa·2AcOH]/0.05 M Tris/0.01% Tween 80 (pH 8.8) for human urokinase (150 international units/ml); 0.02–0.5 mM Spectrozyme PL (*H*-D-Nle-HHT-Lys-pNA·2AcOH)/0.05 M Tris/0.01% Tween 80 (pH 7.4) for human plasmin (0.049 caseinolytic unit/ml); 0.07–3.0 mM Spectrozyme PL (*H*-D-Nle-HHT-Lys-pNA·2AcOH)/0.05 M Tris/0.01% Tween 80 (pH 7.4) for streptokinase (15.1 international units/ml) and human lysine-plasminogen (6.5 μg/ml); 0.03–0.5 mM Spectrozyme PCa [*H*-D-Lys-(γ-CBZ)-Pro-Arg-pNA·2AcOH]/0.03 M Tris/0.03 M imidazole/0.2 M NaCl (pH 8.4) for human protein Ca (3.63 mM) activated by Protac protein C activator (0.25 unit/ml); 0.3–5.0 mM Spectrozyme TRY (CBZ-Gly-D-Ala-Arg-pNA·AcOH)/0.03 M Tris/0.03 M imidazole/0.2 M NaCl (pH 8.4) for bovine trypsin (3.2 units/ml); 0.03–0.5 mM Spectrozyme FXa [MeO-CO-D-cyclohexylglycyl (CHG)-Gly-Arg-pNA·AcOH]/0.05 M Tris/0.01% Tween 80 (pH 8.4) for human factor Xa (20 nkat/ml); 0.007–0.3 mM *N*-succinyl-Ala-Ala-Pro-Phe-pNA/0.1 M Tris/0.01 M $CaCl_2$/0.01% Tween 80 (pH 7.8) for human α-chymotrypsin (0.1 μg/ml).

X-Ray Crystallography. The complex of hirugen and human α-thrombin was prepared as reported (23) and the ternary complex was generated from it by addition of a 10-fold molar excess of CtA. X-ray diffraction data were collected with a Rigaku RU200 rotating anode source and a Siemens multiwire area detector. The detector swing angle was 15°, the scan range was 0.2° per frame, each frame was collected for 90 s, and the diffraction was recorded to 2.3-Å resolution. The intensity data were processed with the program XENGEN (24) to give integrated intensities. The crystal was found to be isomorphous to that of hirugen–thrombin (23): monoclinic, space group C2, four molecules per unit cell with a = 70.61 Å, b = 72.38 Å, c = 73.35 Å, and β = 101.10°. Data reduction resulted in 14,024 independent reflections from 50,070 collected reflections; removal of weak reflections, with $I/\sigma(I) < 2$, gave a data set of 12,650 unique reflections (79% observed; R_{merge} = 0.043). This data set is complete to 2.5-Å resolution and contains half of the possible reflections between 2.3 and 2.5 Å.

The phases corresponding to the crystal structure of the ternary complex were initially approximated from the thrombin coordinates for the complex of hirugen with thrombin (23). The starting crystallographic R factor [$R = (\Sigma||F_o| - |F_c||) / \Sigma|F_o|$] was 0.32. The first electron density at 2.8-Å resolution showed good density for most of the components; hirugen and CtA were also prominent in the ($|F_o - F_c|$) difference map. The structure was refined by using restrained least-squares methods with PROLSQ (25), in which the dictionary and control files were modified to apply proper geometric restraints to the CtA model. This refinement entailed a series of geometrically "tight–loose–tight" cycles proceeding to individual thermal parameters and gradually increased resolution (2.8, 2.5, 2.3 Å), with water molecules being introduced at 2.5 Å. The final structure has R = 0.138 (7.0–2.3 Å) with an average thermal parameter of 29 $Å^2$, 239 water molecules, and 11,777 reflections. The rms deviations from modeled bond lengths and angles are 0.019 Å and 2.8°, and 98% of the residues are in conformationally allowed regions.

Synthetic Chemistry. The scheme used for synthesis of CtA, with reagents and conditions, is depicted in Fig. 2. Complete experimental details for the procedures will be reported separately (details available from B.E.M. on request). Reaction products were characterized by ^1H NMR (400 or 500 MHz) and fast atom bombardment mass spectrometry. Reaction yields are for unoptimized procedures and isolated, purified materials. Two diastereomers (≈1:1 ratio) were carried from cyanohydrin **2** up to the Dess–Martin oxidation (step v).

RESULTS

Structure of the Thrombin–CtA Complex. We determined the molecular structure of a complex involving CtA, hirugen, and thrombin by x-ray crystallography. The first electron-density map based on phases of thrombin coordinates showed hirugen occupying the exo site, in a conventional manner (23, 27), and CtA occupying the active site. This result unequivocally establishes the chemical structure of CtA, as shown in Fig. 1*a*, which corroborates the structural correction put forth by Hagihara and Schreiber (7). Furthermore, we now possess the structural details reflective of molecular recognition within the enzyme active site (Figs. 3–5).

Clearly, the Pro-Arg unit of CtA interacts with the enzyme at the S_2 apolar and S_1 specificity sites, respectively, similar

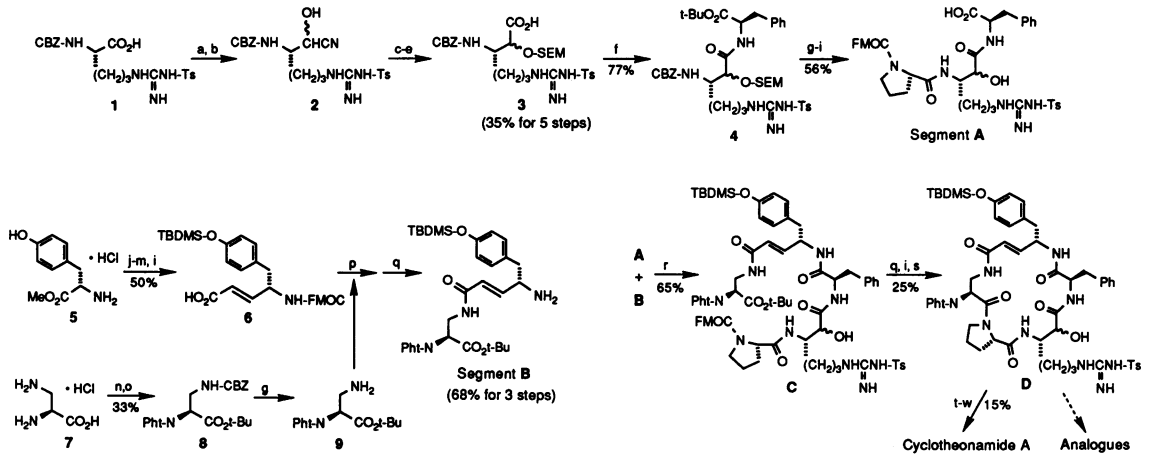

FIG. 2. Scheme for synthesis of CtA and its analogues. The following reagents were used: a, (*i*) (1-imidazolyl)$_2$C=O, (*ii*) *i*-Bu$_2$AlH; b, KCN; c, HCl, MeOH; d, SEM Cl, 2,6-lutidine; e, LiOH; f, D-PheO-*t*-Bu, HOBt, DCC; g, Pd(OH)$_2$, H$_2$; h, FMOC-L-Pro, HOBt, DCC; i, CF_3CO_2H; j, FMOC Cl, K_2CO_3; k, TBDMS Cl, imidazole; l, *i*-Bu$_2$AlH; m, Ph_3P=$CHCO_2$-*t*-Bu; n, published procedure (26); o, *N*-carbethoxyphthalimide; p, HOBt, EDC·HCl; q, Et_2NH; r, BOP Cl, Et_3N; s, BOP Cl, 4-dimethylaminopyridine; t, $N_2H_4·H_2O$, 4-pentenol; u, HCO_2Et; v, Dess–Martin reagent; w, HF. Me, methyl; Et, ethyl; Bu, butyl; Ph, phenyl; CBZ, carbobenzoxy; FMOC, fluorenylmethoxycarbonyl; Ts, *p*-toluenesulfonyl; SEM, trimethylsilylethoxymethyl; TBDMS, *tert*-butyldimethylsilyl; Pht, phthaloyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; EDC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide; BOP Cl, bis(2-oxo-3-oxazolidinyl)phosphinic chloride.

to the Pro-Arg of PPACK (11, 12). In this vein, the guanidinium group of Arg participates in a doubly hydrogen-bonded ion pair with Asp^{189} of thrombin and the -NH-C-C(O)-Pro-Arg- segment forms a hydrogen-bonded two-strand antiparallel β-sheet with Ser^{214}–Gly^{216} (Fig. 4). The two carbonyl groups of the α-keto amide are oriented at a dihedral angle of 109° (Fig. 5), akin to the dicarbonyl conformation in FK-506, a macrocyclic α-keto amide immunosuppressant, when it is complexed to the immunophilin FKBP (28). However, the α-keto amide of CtA is involved in a complicated hydrogen-bonded, tetrahedral intermediate (hemiketal) that resembles a transition state for peptide hydrolysis (Fig. 5). The Ser^{195} O$^{\gamma}$-C^2 bond distance is 1.8 Å, which compares to a Ser^{195} O$^{\gamma}$-carbonyl bond distance in PPACK–thrombin of 1.6 Å (11, 12) (C-O single bond distance in an aliphatic ether, ≈1.45 Å). In the tetrahedral array, the O$^{\gamma}$ atom impinges orthogonally on the α-keto group from the *re* face (∠ O$^{\gamma}$-C^2-O^2 = 86°; ∠ C^1-C^2-O$^{\gamma}$ = 93°; ∠ C$^{\alpha 2}$-C^2-O$^{\gamma}$ = 97°); also, the keto oxygen (O^2) makes a bifurcated hydrogen bond with thrombin.

As for other features, the CtA molecule adopts a relatively "open" conformation for the 19-membered ring with the proline ring orthogonal to the macrocyclic ring and one transannular hydrogen bond between O^5 and N^{11} (Fig. 1*a*; 3.1 Å). The Tyr^{60A}, Trp^{60D}, and Leu^{99} residues define a hydrophobic pocket that envelops the hydrocarbon portion of the proline of CtA. The aromatic groups of CtA engage in an aromatic stacking chain that encompasses Tyr^{60A} and Trp^{60D} of thrombin (Fig. 4; Table 1). This represents an unusual interaction for a thrombin inhibitor ligand because Trp^{60D} of the insertion loop, from the indole face opposite to the proline pocket, directly associates with CtA via the hydroxyphenyl group. An interaction also exists between the D-Phe of CtA and Leu^{40}-Leu^{41}. Other close contacts are limited (Table 1). Significantly, the C^{13}-C^{14} double bond of CtA is not involved in covalent attachment to the enzyme, but its proximity to Glu^{192}-Gly^{193} could be relevant to binding.

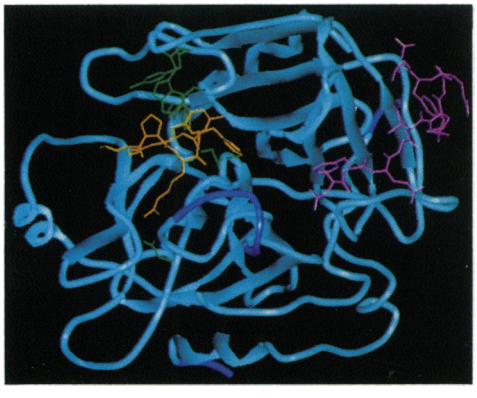

FIG. 3. View of the ternary complex involving thrombin (blue), CtA (orange), and hirugen (magenta). The backbone of thrombin is shown in a solid-ribbon format, with the following side chains in green: His^{57}, Tyr^{60A}, Trp^{60D}, Asp^{102}, Asp^{189}, Ser^{195}. Amino acids missing because of disorder have been added to the backbone (darker blue).

Enzyme Inhibition Studies. We investigated the action of CtA as an inhibitor of human α-thrombin and nine related serine proteases by enzyme kinetics (Table 2). The reversible, active-site-directed inhibitors GYKI-14766 (18, 19) and argatroban (29, 30) were also examined as reference standards. As such, our compilation affords a unique set of inhibition data for comparison. CtA exhibits only moderate, competitive inhibition against thrombin (K_i = 0.18 μM), but it is a much better inhibitor of trypsin (K_i = 0.023 μM) and streptokinase (K_i = 0.035 μM). Its inhibition of thrombin is within the range of its inhibition of urokinase (K_i = 0.37 μM) and plasmin (K_i = 0.37 μM). GYKI-14766 has structural elements similar to those of CtA, including the possibility of forming a tetrahedral intermediate, although it is noncyclic and has a D-Phe group that affords an aromatic stacking interaction with Trp^{215} (11, 12). Our data (Table 2) indicate that GYKI-14766 is a potent, competitive inhibitor of thrombin (K_i = 0.016 μM) and trypsin (K_i = 0.032 μM); thus, it is not selective in this respect. By contrast, argatroban is an exceedingly selective inhibitor of thrombin (K_i = 0.0081 μM). Interestingly, CtA is ≈10 times less effective than GYKI-14766 as a thrombin inhibitor, although both contain similar P_2-P_1 recognition components.

Synthetic Chemistry. The lack of potency and selectivity of CtA for thrombin could be addressed by a structure–function study with cyclotheonamide analogues. To this end, we developed synthetic methodology, based on a convergent [2 + 3] fragment-condensation route (Fig. 2). In the synthetic scheme, the requisite protected amino acid starting materials were processed and converted into two segments, **A** and **B**, which were coupled in 65% yield to give intermediate **C**.

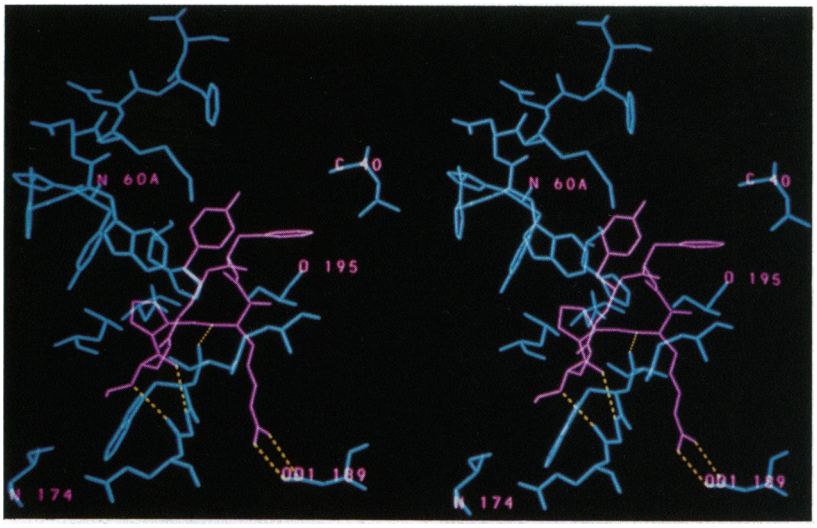

FIG. 4. Stereoview of CtA bound in the active-site cleft of thrombin. CtA, pink; thrombin, blue; selected hydrogen bonds, broken yellow.

After selective removal of the terminal protecting groups on C, the critical macrocyclization was effected in 65% yield under high-dilution conditions (0.001 M). Intermediate macrocycle **D** was then processed in four steps to CtA, which was isolated and purified by HPLC (trifluoroacetate salt). The synthetic CtA was identical to the natural product by 500-MHz ^{1}H NMR, HPLC, TLC, and fast atom bombardment mass spectrometry. Our chemical protocol, secured by the successful total synthesis of CtA, provides a vehicle for the systematic preparation of analogues.

A total synthesis of cyclotheonamide B, involving a different approach and different chemistry, was recently disclosed by Hagihara and Schreiber (7). Also, two syntheses of molecular fragments were reported recently (31, 32).

DISCUSSION

At the outset of our work we entertained the notion that CtA, as a cyclic ligand for thrombin, might reveal new modes of molecular recognition in the active-site cleft, particularly regarding the unique Tyr^{60A}-Thr^{60I} insertion loop of thrombin. Although the Pro-Arg motif of CtA was expected to interact in a manner similar to that of PPACK, as observed in the crystallographic structure of the PPACK–thrombin complex (11, 12), the cyclic organization and additional substituents of CtA offered intriguing possibilities for new interactions.

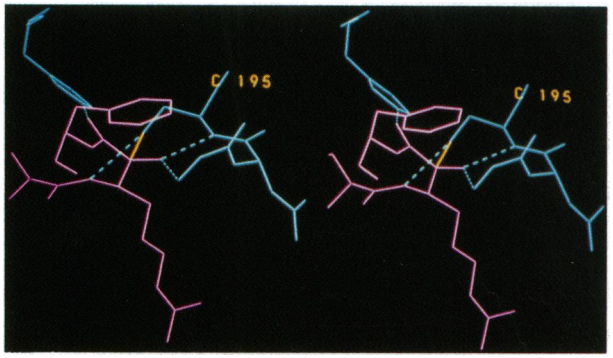

FIG. 5. The α-keto amide group of CtA within the thrombin complex. Stereoview of the structure in the vicinity of the α-keto amide of CtA with four hydrogen bonds (broken blue); CtA, pink; thrombin, blue; transition-state tetrahedral intermediate bond (1.8 Å), yellow.

From x-ray crystallography, we now appreciate that the CtA–thrombin complex exhibits conventional Pro-Arg molecular recognition, a tetrahedral transition-state analogue structure involving the ketone carbonyl and Ser^{195}, and an intermolecular aromatic stacking interaction between Trp^{60D} of the insertion loop and the hydroxyphenyl of CtA. There is no covalent linkage between the enzyme and the potentially reactive vinyl group of CtA (C^{13}-C^{14}; Fig. 1*a*).

There has been a paucity of studies on the α-keto amide functionality in the design of enzyme inhibitors, although α-keto esters and other activated carbonyl derivatives have received substantial attention (13–15, 33–35). Besides the naturally occurring CtA described here, some α-keto amide inhibitors of proteases have recently been described (33–35). In this context, we should also consider the α-keto amide immunosuppressant ligands, such as FK-506, which complex with immunophilins and evince peptidylprolyl *cis–trans*-isomerase (PPIase) inhibitory activity. It has been suggested that the α-keto amide group in these complexes may mimic a "twisted amide" transition-state structure, with a dihedral angle between carbonyl groups (C^8-C^9) of 95° to 100° (30, 36, 37). Although complexes for such macrocycles bound to immunophilins have been observed crystallographically (30, 38), our study with CtA and thrombin represents the first visualization of an α-keto amide transition-state complex for a serine protease. Comparing systems, there is a distinct difference in structure of the α-keto amide portion of the ligands, as might be expected from their difference in function: PPIase inhibition vs. serine protease inhibition.

The enzyme inhibition studies reported here have profiled the properties of three inhibitor structures—CtA, GYKI-14766, and argatroban—with 10 important proteolytic enzymes. This forms a sound basis for characterization of

Table 1. Close van der Waals contacts between CtA and thrombin

CtA	Thrombin	Distance, Å
Pro CG	Tyr^{60A} CZ	3.6
Pro CG	Trp^{60D} CH2	3.7
D-Phe CZ	Leu^{40} CD2	3.6
D-Phe CE2	Leu^{41} C	3.5
D-Phe CZ	Gly^{193} CA	3.1
C^{14} (Fig. 1*a*)	Trp^{60D} CZ2	3.5
C^{14}	Glu^{192} CG	3.8

C, carbonyl carbon; CG, γ carbon; CE2, ε2 carbon; CA, α carbon; CD2, δ2 carbon; CZ, ζ carbon; CZ2, ζ2 carbon; CH2, η2 carbon.

Table 2. Characterization of CtA as an inhibitor of thrombin and related serine proteases

Enzyme	CtA	Argatroban	GYKI-14766
Thrombin	0.18 ± 0.04 (6)	0.0081 ± 0.0011 (4)	0.016 ± 0.005 (3)
Plasmin	0.37 ± 0.08 (5)	400 ± 40 (3)	0.70 ± 0.09 (4)
Kallikrein	0.51 ± 0.21 (4)	670 ± 150 (4)	0.98 ± 0.23 (3)
tPA	6.5 ± 1.8 (4)	370 ± 100 (3)	1.7 ± 0.4 (4)
Urokinase	0.37 ± 0.13 (4)	Inactive at 500 μM (2)	2.3 ± 0.7 (3)
Streptokinase	0.035 ± 0.003 (4)	Inactive at 500 μM (2)	1.3 ± 0.02 (4)
Factor Xa	130 ± 40 (3)	390 ± 140 (3)	95.0 ± 25.0 (6)
Trypsin	0.023 ± 0.007 (3)	7.9 ± 0.3 (3)	0.032 ± 0.022 (4)
Chymotrypsin	6.9 ± 2.8 (4)	Inactive at 500 μM (2)	Inactive at 100 μM (2)
Protein Ca	3.1 ± 0.8 (5)	Inactive at 500 μM (2)	2.0 ± 0.5 (4)

K_i values (μM; mean ± SE) are given. Number of independent experiments, each done in duplicate, is in parentheses. tPA, tissue-type plasminogen activator.

analogous thrombin inhibitors in future endeavors. CtA is not a potent, selective thrombin inhibitor, and its substantial inhibition of streptokinase (5-fold better than thrombin), urokinase, and plasmin is therapeutically undesirable. Such data indicate that CtA is not an especially effective ligand for the thrombin active site and suggest that the auxiliary groupings on CtA may be less than optimally disposed within the complex.

In this situation, it is imperative to assess the x-ray crystallographic structure with respect to the enzyme inhibition data (Table 2). Although interactions due to the α-keto amide transition-state analogue format, the S_1 specificity pocket, and the β-sheet hydrogen bonds dominate the binding of CtA, binding interactions by the remainder of the macrocycle are not strongly supportive. The hydroxyphenyl substituent on CtA is not optimally located relative to Trp^{60D} (or Tyr^{60A}) of the insertion loop, and the phenyl substituent on CtA contributes little advantage. In addition, the hydrophilic formamide group, although involved in a β-sheet hydrogen bond via the NH, does little to satisfy a hydrophobic region, near Trp^{215}, that is occupied by the D-Phe of PPACK (11, 12). The enzyme inhibition data mirror the deficiencies of molecular recognition for CtA–thrombin inferred from the crystallographic results. We envision modifying the structure of CtA to obtain improved thrombin inhibitors.

Overall, our work not only enhances understanding of intermolecular interactions between macrocyclic inhibitors and their binding proteins, but it also holds special significance for the design and synthesis of thrombin inhibitors as potential antithrombotic drugs.

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